AWARD NUMBER: W81XWH-15-1-0416

TITLE: Biofluid-Based Detection of the Migration Switch in Prostate Cancer to Predict Metastatic Disease

PRINCIPAL INVESTIGATOR: Lian Willetts

CONTRACTING ORGANIZATION: University of Alberta
Edmonton T6G 1K8
Alberta, Canada

REPORT DATE: September 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
September 2016

2. REPORT TYPE
Annual

3. DATES COVERED
19 Aug 2015 - 18 Aug 2016

4. TITLE AND SUBTITLE
Biofluid-Based Detection of the Migration Switch in Prostate Cancer to Predict Metastatic Disease

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0416

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Willetts L., Pink D., Sosnowski D., Paproski R.

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Alberta
Augustana, Campus Saint-Jean
RSO
222 Campus Tower
8625-112 street, Edmonton
Alberta, Canada

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

- We discovered two new potential biomarkers in additional to CD151.
- The optimization protocols for microflow cytometry has more stringent requirement than regular whole cell based flow cytometry. The development of our assay parameters established standards for future clinical assay development and current liquid biopsy collection and handling protocols.
- The development of our test has optimized no fewer than 30 parameters spanning clinical sampling and storage to antibody dilution and conjugation. To improve the time for actual analysis, we have isolated a pre-diagnosis cohort of ~100 patients (Negative and positive for PCa) that are balanced for age and PSA (<10ng/mL) at time of biopsy. This small cohort will be used for preliminary assessment of new potential biomarkers and antibody variants (i.e. different epitopes) for any particular biomarker.
- I received an invitation to be a speaker at the Alpbach Technology Forum (Austria) - New enlightenment and the Austrian Falling walls 2016 competition will take place. My work from the competition were highlighted in the President's 'report at the university governance committee. An open doors event in which I had the opportunity to present my work. There is a collaboration with Department of genetics, Harvard Medical School (institute of RNA medicine) - Using Dr. Lewis' miRNA and chicken embryo platform in researching breast cancer. The collaborator is a HMS's scientist, who researches miRNA medicine/therapeutics and has discovered one particular RNA that may have a role in breast cancer. Another in-house collaboration was developed with a laboratory in oncology studying osteosarcoma using Dr. Lewis' in house imaging platforms.

15. SUBJECT TERMS

Metastasis, motility switch, indicator, prostate cancer, liquid biomarkers

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	11	

Table of Contents

Page

ANNUAL TECHNICAL REPORTING REQUIREMENTS

1. INTRODUCTION:

Many newly diagnosed cases of prostate cancer are considered low risk clinically and destined to remain asymptomatic for a patient's life as they are associated with the presence of low grade tumors in biopsy samples, but metastasis may occur early and originate in localized disease that is not histologically aggressive. The presence or absence of metastases is the only individual prognostic factor in multivariate analysis of prostate cancer outcomes. While the five year survival for localized prostate cancer in North America is close to 100%, the survival rate drops to 31.9% for metastatic disease. Being able to accurately and non-invasively monitor localized disease longitudinally will allow patients to avoid the side effects and morbidity of definitive treatment (*e.g.* surgery or radiation). Metastasis represents the most lethal aspect of prostate cancer, and thus there is an urgent need for biological indicators to predict metastasis. This topic is not only of high interest for basic prostate cancer research, but also opens novel possibilities for establishing therapies that target metastatic prostate cancer.

2. KEYWORDS: Metastasis, prostate cancer, liquid biomarkers, cancer microparticles and motility indicators.

3. ACCOMPLISHMENTS:

Specific Aim 1: Internal validation of biofluid-based and surgical tissue-based detection of the migratory switch (CD151 and CD151^{free}) as a clinical predictor of patient outcome

1. Accomplished Training-specific tasks:

- Workshop: 3rd May, 2016: Pre-Meeting Education Day program in two parallel sessions: Novel developments isolation and characterization of extracellular vesicles and Extracellular vesicle therapeutic
- Presentation: Willetts L., Breaking down the wall of Prostate Cancer metastasis. Alpbach Technology Symposium, Aug. 2016, Alpbach, Austria
- Presentation: Willetts L., Konstantin Stoletov, Emma Woolner and John Lewis. Intravital discovery of miRNA drivers of metastatic cascade in human cancers. Prostate Cancer Collaborative Research 2015 Symposium, Nov. 2015, Brisbane, Australia
- Presentation: Willetts L., Breaking the Wall of Prostate Cancer Metastasis. Falling Walls Conference, Berlin, Germany, Nov.8-9th 2015
- Presentation: Pink D., Willetts L., Sosnowski D., Lee R., Chepesh A., Paproski R., Zijlstra A., Lewis J.D., Development of an extracellular vesicle micro-flow cytometry assay for prostate cancer prognosis. 8th International TSPAN Scaffolding Research Conference, June 2015, Nashville, TN, USA

I presented our research project at the 2015 APCaRI symposium. APCaRI consist of patients, physicians, and scientists. The biannual APCaRI symposium is a great way to disseminate interest to communities that are Western Canada region. Our initiative hosts 2 provincial meetings every year. Average attendance is 48 participants including Clinicians, Scientists, Pathologists, and Trainees, members of support groups, philanthropists and Clinical Research Personnel. During the meetings, members of the team present their latest findings in prostate cancer or biomarker-associated studies (~16 talks per meeting), trainees submit abstracts for posters and oral presentations. These meetings enhance the knowledge about provincial progress in prostate cancer research, current clinical trials, and potential collaborations and grant applications. During and after the meeting, the team has established multiple collaborations with different stakeholders including clinicians and scientists. At least 6 National and international, multidisciplinary grant applications have been submitted with various team members. Trainees have become more involved and enthusiasts about translational research and clinical research. Philanthropists are satisfied with the progress made by the team and are continuously fundraising for our efforts.

I also presented at the Prostate Cancer Collaborative Research (PCCR) Symposium 2015 in Brisbane, Australia which gave me opportunities to share my research progress with our Australian counterparts in scientific research on prostate cancer. In November 2015, based on the research results we obtained on the project, I competed and presented at the 2015 Falling Walls conference. The Falling Walls Lab is an international competition that challenges participants to showcase how their research is redefining their respective fields and breaking down the walls to the next major scientific breakthrough. The University of Alberta is one of 34 approved international labs, and the Sept. 30 event saw 16 outstanding examples of research in science and society. I was awarded 1st place in the University of Alberta Lab. The international Labs and the Falling Walls Conference is a global scale event that takes place in Berlin, Germany on the 9th of November each year. The Finale gathers 100 winners of the international Labs. I was awarded 2nd place in the Falling Walls Lab Finale in Berlin representing Dr. John Lewis' lab and our project by presenting: "Breaking the Walls of Prostate Cancer Metastasis". The Falling Walls Lab is a great way to disseminate our research to an international community of people with various background and interest.

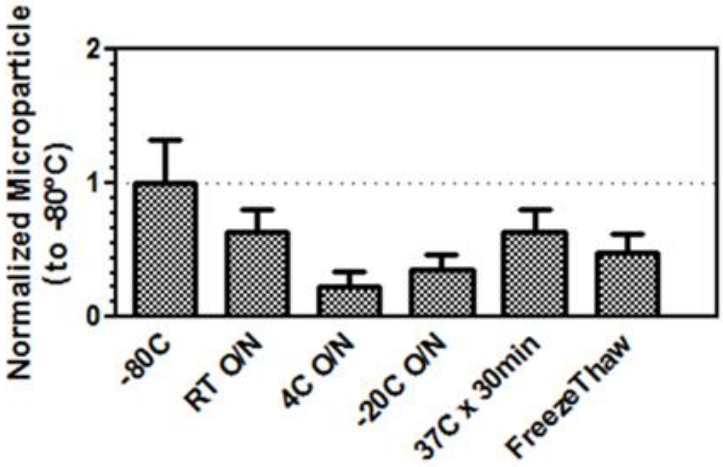
2. **Accomplished Research-Specific tasks:**

Major Task 1: Internally validating biochemical detection of CD151^{free+} PSMA^{+} cancer microparticles (CMP) in biofluids as a clinical predictor of patient outcome.

Subtask 1: Training the micro-flow cytometer Apogee, and optimization of biofluid-based microparticle detection. Samples used: Vanderbilt retrospective surgical cohort (total 500 patient samples). Internal validation of biofluid-based and surgical tissue-based detection of the migratory as a clinical predictor of patient outcome switch. Major task1: Internally validating biochemical detection of CD151+PSMA+CMP in biofluids and a clinical predictor of patient outcome.

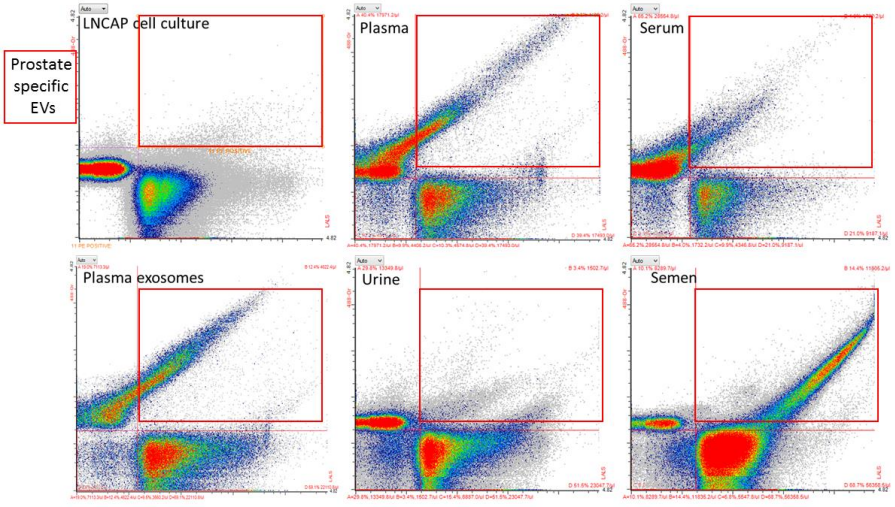
- Training: The micro-flow cytometer Apogee, and optimization of biofluid-based microparticle detection.
- Research milestone achieved: Optimized clinical sample handling and clinical assay development

Figure 1. Delaying processing beyond 2hr or storing plasma at temperatures higher than -80C significantly reduces the frequency of PSMA positive microparticles.



- Research milestone achieved: Detected prostate specific extracellular vesicles in various clinical samples

Figure 2. PSMA positive CMP can be detected in various biological fluids.



- Research milestone achieved: 2 more motility indicators have been discovered during this period of time and we validated them in 71 patients' cohort

Figure 3. PSMA antibodies with similar epitopes detect elevated PSMA positive CMP in plasma from metastatic and non-diseased samples.

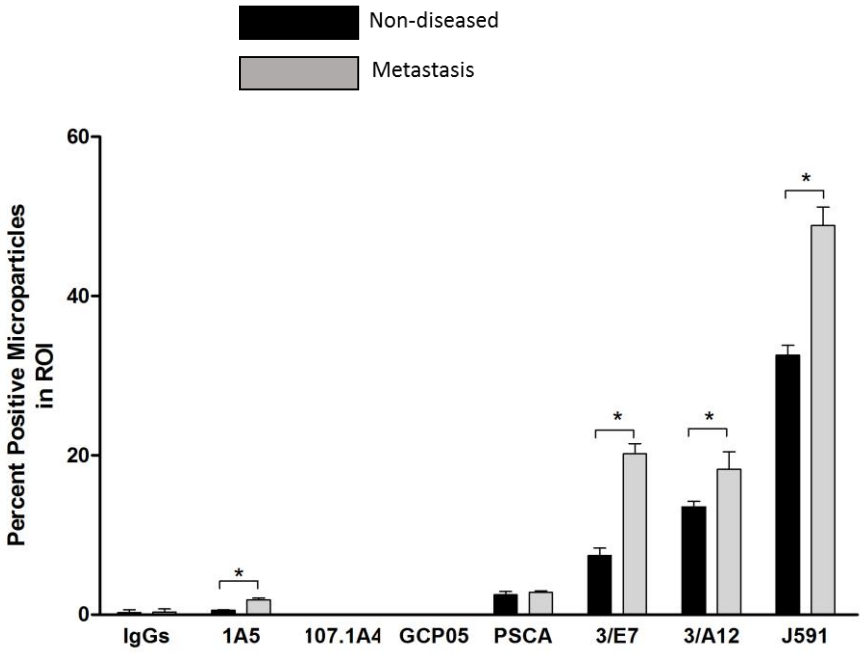
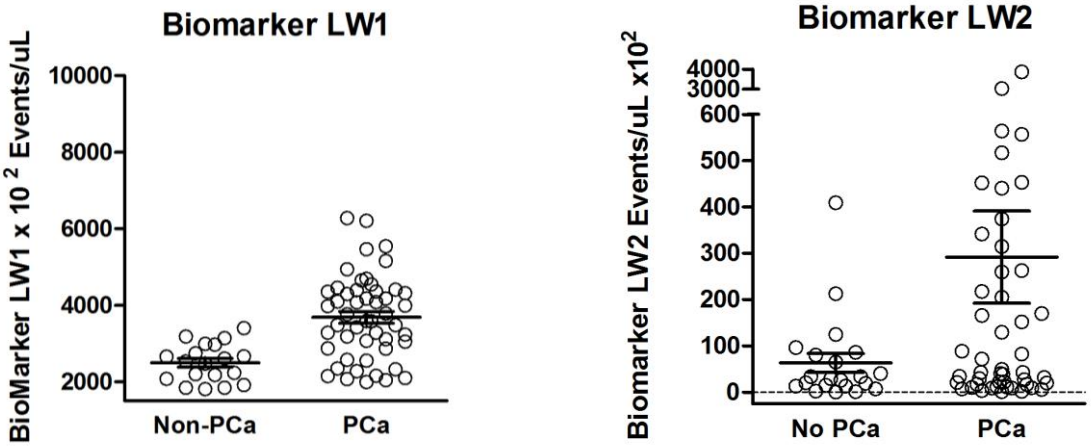


Figure 4. Two additional biomarkers LW1 and LW2 have diagnostic potential using human in plasma samples

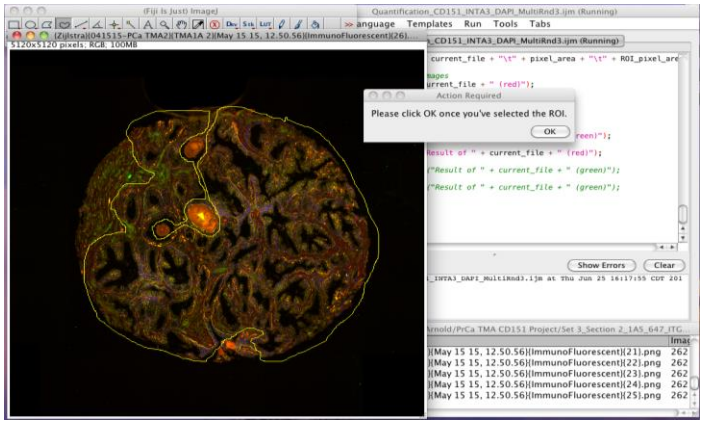


- Biomarker LW1 was selected based on a search of the literature and biomarker. LW2 was selected based on a screen for genetic markers of metastatic PCa. Both biomarkers were analyzed using micro-flow cytometer Apogee; clinical designation of patient sample was made by a trained pathologist using core biopsy samples of prostate. Flow analyses were compared (No disease vs. PCa) using Students t test, 2Way, unpaired; both biomarkers were significantly elevated in the PCa patients $p>0.05$ (LW1, $p<0.0001$; LW2, $p=0.0289$).

Major task 2: Histological analysis of CD151 status in biopsy and surgical tissues for internal validation
Subtask 1: Tissue staining with antibodies against CD151 (free and anchored) and integrin α3. Samples used: Vanderbilt retrospective surgical cohort (total 500 patient samples)

- Optimization for Immunohistochemistry of CD151 Immunohistochemistry:
  Staining for CD151 and CD151 free, integrins α3 and α6, CK and collagen has been optimized as two multiple colour fluorescence IHC protocols. Staining of Vanderbilt operational TMA (100 pts) using both multiple colour panels has been done and data has been analyzed (Fig. 5).

Figure 5: Samples of multi-colour IHC stained biopsy tissue.



As a collaborative effort between Prostate Cancer Canada and the Terry Fox Research Institute, the CPCBN (Canadian Prostate Cancer Biomarker Network) granted our team access to their Tissue Microarray (TMA). An optimization TMA was delivered to our team, stained and sent back to CPCBN for evaluation. The team is satisfied with the quality of the staining and a validation test cohort TMA with 250 radical prostatectomy samples was recently sent to us. Once the first 250 patients are analyzed, a large validation TMA with 1250 radical prostatectomies will be supplied to us at no cost. Optimization slide from CPCBN was stained and images from it were returned for evaluation.

- Retrospective validation of Vanderbilt cohort TMA Progress:
  100 case operational TMA (Normal, BPH, HGPIN, all GS patterns) currently completed and stained, data analyzed. Significant differences detected for several of the biomarkers in this initial cohort.
  664 cases with complete case history collated and annotated, 125 confirmed biochemical recurrence. TMA will be completed in Q1 2017 with staining/analysis to follow.
  Comparison is made to 150 age-matched normal males and 300 patients with other cancers (Renal, Bladder)

Subtask 2: Stained sections will be scored in a double-blinded manner suing ordinal vales to denote staining intensity, location and area

- Data analysis and interpretation
  Field mapping approach to assess multiplex staining protocols has been developed and validated in the operational TMA. Operational TMA analysis complete.

Specific Aim 2: External validation of the biofluid-based detection of migratory switch ($CD151^{free+}$ $PSMA^{+}$CMP) as a clinical predictor of patient outcome.

Major Task 1: Preliminary cohort with samples from 100 non-cancer, 100 localized and 100 metastatic patients has been received and will be analyzed shortly

3. **In the next reporting period I plan to accomplish the following:**

- To improve the power of our prediction platform by combining individual biomarkers with significant prognostic potentials. I will be focus on developing and optimizing clinical protocol for analyzing multiple biomarker signals simultaneously using patients' serum, plasma and urine samples from the proposed cohorts.
- To discovery and validate other motility indicators.
- To analyze and score the TMA sections.

4. IMPACT:

- We discovered two new potential biomarkers in additional to $CD151^{free}$, and validated their diagnostic and prognostic potential in 71 patients cohort. The discovery and validation of additional biomarkers have significantly improved the accuracy and sophistication of our platform. A multivariate analysis platform based on our motility indicators on CMP in plasma have the potential to offer additional accurate predictive information to physicians and patients.
- The optimization protocols for microflow cytometry has more stringent requirement than regular whole cell based flow cytometry. The development of our assay parameters established standards for future clinical assay development and current liquid biopsy collection and handling protocols.
- Adoption of new practices: As indicated by Figure 1. The development of our test has optimized no fewer than 30 parameters spanning clinical sampling and storage to antibody dilution and conjugation. To improve the time for actual analysis, we have isolated a pre-diagnosis cohort of ~100 patients (Negative and positive for PCa) that are balanced for age and PSA (<10ng/mL) at time of biopsy. This small cohort will be used for preliminary assessment of new potential biomarkers and antibody variants (*i.e.* different epitopes) for any particular biomarker.
- I received an invitation to be a speaker at the Alpbach Technology Forum (Austria) - New enlightenment (Aug 25-27, 2016) and the Austrian Falling walls 2016 competition took place. My work from the competition were highlighted in the President's 'report at the university governance committee, which is an open doors event in which I had the opportunity to present my work to a broad audience within the university community. There is a collaboration with Department of genetics, Harvard Medical School (institute of RNA medicine) - Using Dr. Lewis' intravital image platform in researching breast cancer metastasis. The collaborator is a HMS's scientist, who researches miRNA medicine/therapeutics and has discovered one particular RNA that may have a role in breast cancer. Another in-house collaboration was developed with a laboratory in oncology studying osteosarcoma using Dr. Lewis' intravital imaging platforms.

5. CHANGES/PROBLEMS

The acquisition of a large enough metastatic cohort was delayed. The pulling, and aliquoting of the cohort took longer than expected, as did the linkage of the clinical data to the samples. We have now just received serum and clinical data from the metastatic cohort and hope to finish the analyses in the coming weeks. This delay however allowed us to examine some potential biomarkers in a pre-diagnosis cohort, acquired and maintained by our local APCaRI Registry and Biorepository. This has proved fruitful (see Figure 4.) in the identification of two new potential biomarkers of PCa. As part of the research into biomarkers of PCa, we, and others, fully realize that one or even two markers will not be sufficient in accurately stratifying a complicated disease such as cancer. Rather a panel of biomarkers in combination with clinical parameters such as age, genetics, and race will provide a more useful and robust clinical assessment of a patients overall risk for PCa.

6. PRODUCTS:

Conference papers, and presentations:

- Willetts L., Breaking the Wall of Prostate Cancer metastasis. Alpbach Technology Symposium, Aug. 2016, Alpbach, Austria
- Willetts L., Konstantin Stoletov, Emma Woolner and John Lewis. Intravital discovery of miRNA drivers of metastatic cascade in human cancers. Prostate Cancer Collaborative Research 2015 Symposium, Nov. 2015, Brisbane, Australia
- Willetts L., Breaking the Wall of Prostate Cancer Metastasis. Falling Walls Conference, Nov.8-9th 2015, Berlin, Germany (https://www.youtube.com/watch?v=FxL2eopFcHw)
- Pink D., Willetts L., Sosnowski D., Lee R., Chepesh A., Paproski R., Zijlstra A., Lewis J.D., Development of an extracellular vesicle micro-flow cytometry assay for prostate cancer prognosis. 8th International TSPAN Scaffolding Research Conference, June 2015, Nashville, TN, USA
- Pink D., Willetts L., Sosnowski D., Lee R., Chepesh A., Paproski R., Zijlstra A., Lewis J.D., Development of an extracellular vesicle micro-flow cytometry assay for prostate cancer prognosis. International Society of Extracellular Vesicles (ISEV), May 2016, Rotterdam, Netherlands

Publications:

- Willetts L., David Bond, Konstantin Stoletov, John D. Lewis. Quantitative Analysis of human Cancer Cell Extravasation Using Intravital Imaging, Department of Oncology, University of Alberta (2016) Vol. 1458, The Tumor Microenvironment, Methods Molecular Biology.

Website(s) or other Internet site(s): www.APCaRI.ca:

The APCaRI Web Portal and Knowledge Exchange Hub provides information to our collaborators, stakeholders and end-users, as well as the general public. The exchange hub serves as a mechanism to collaborate within the group and

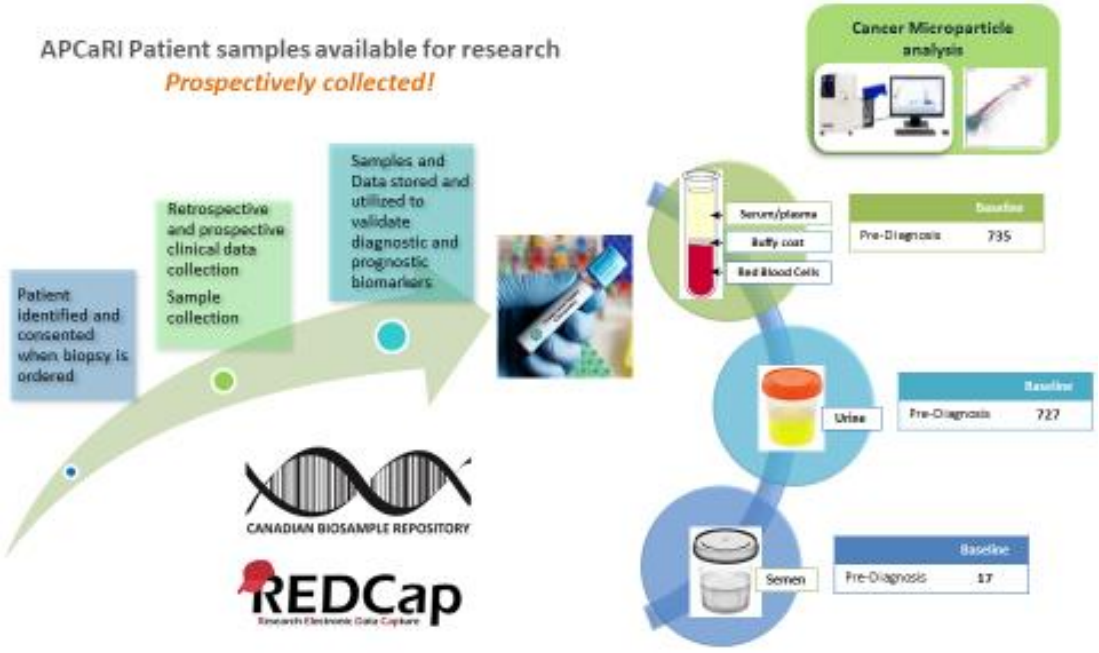
disseminate research outputs. The blog and discussion areas facilitate information exchange with end-users. Fundraising initiatives are involved. Enhanced exposure of the team, the research performed and the impact we are aiming to achieve. Participation from the different audiences through the newsletter and contact us section. From April 1, 2015 to March 31, 2016, there were 11,269 visits to the APCaRI webpage with 19,242 page views. 79% of the visitors were new, indicating great exposure of APCaRI at national and international levels. Since launch in February 2015, over 900 people from all over the world have visited it. We have received multiple applications for jobs, to be enrolled in graduate programs and to participate in our studies.

Other Products:

Our team has developed and maintained infrastructure in Edmonton and Calgary to longitudinally collect: 1) biospecimens, including blood, urine, semen, tissue (when applicable); 2) clinical information pertaining to the management of prostate cancer and other health-related events; and 3) patient-reported health quality-of-life and symptoms from patients with confirmed prostate cancer across Alberta. For the reporting period, 1140 participants have been enrolled in the study and provided baseline blood, urine, and or semen and shared associated health information to support health research that makes use of biospecimens in conjunction with clinical and patient-reported outcome data to discover novel molecular markers relevant to the care and management of patients. These patients will visit participating clinics once a year for 5 years to donate biospecimens and complete quality of life questionnaires. The goal is to enroll over 35oo participants in the next 3 years for a total of 5000 patients at the end of year 5. The samples will be used to validate the team's promising biomarkers for prostate cancer diagnosis and biomarkers of aggressive disease that could aid in treatment decisions.

A subset of samples were distributed to a collaborator in Quebec - results from the analysis generated significant hypothesis that led to a grant application to Prostate Cancer Canada. Results from the application will be known in June 2016. Biospecimens from 500 participants were analyzed for a diagnostics biomarker and results are summarized in a manuscript that will be submitted for publication on May 2016.

Figure 6. The Alberta prostate cancer biorepository



7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- The PI is Dr. Lian Willetts.
- Other individuals worked on the project are:
    1. Name: Pink D.
       Role: clinical coordinator
       Nearest person month worked: 12 months
       Contributions to Project: Dr. Pink trained me on the proper use of the microflow machine. He is the interface between our research and the patients.
    2. Name: Sosnowski D.
       Role: Technician
       Nearest person month worked: 12
       Contribution to Project: Mrs. Sosnowski performed antibody conjugation, general maintenance of the microflow machine and assisted in analysing patients samples.
    3. Name: Paproski R
       Role: machine learning expert
       Nearest person month worked: 12
       Contribution to project: Dr. Paproski performed work in designed machine learning algorithms for data analysis.

There been no change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period. Medical Oncologists, Radiation Oncologists, Urologists are helping by identifying eligible candidates for our Registry and Biorepository and referring them to our Clinical Research Coordinators and Research Nurses who will obtain informed consent and will coordinate sample and data collection. Pathologists are assisting in identification of areas with tumour for our biomarker discovery studies.

8. SPECIAL REPORTING REQUIREMENTS: N/A

9. APPENDICES: N/A